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Supplementary appendix

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Supplementary Appendix

SARS-Cov-2 specific antibody and T cell responses 1 year after infection in individuals recovered from COVID-19: a longitudinal cohort study

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Methods supplement

Data and blood sample collection at follow-up visit

At 12-month follow-up visit, survivors were interviewed with questionnaires on symptoms and venous blood was collected.

Inclusion and exclusion criteria

Inclusion criteria:

All patients with laboratory confirmed COVID-19 who were discharged from Wuhan Research Center for Communicable Disease Diagnosis and Treatment, Chinese Academy of Medical Sciences (Wuhan, China) between Jan 7 and May 29, 2020.

Exclusion criteria

(1) those who died before the follow-up visit,

(2) those for whom follow-up would be difficult owing to psychotic disorder, dementia, or re-admission to hospital attributed to underlying diseases,

(3) those who were unable to move freely due to concomitant osteoarthropathy or immobile before or after discharge due to diseases such as stroke or pulmonary embolism,

(4) those who declined to participate,

(5) those unable to be contacted, and

(6) those living outside of Wuhan or in nursing or welfare homes.

Case definition

The disease severity was characterized by the highest seven-category scale during the hospital stay (termed the severity scale), which consisted of the following categories: 1, not admitted to hospital with resumption of normal activities; 2, not admitted to hospital, but unable to resume normal activities; 3, admitted to hospital but not requiring supplemental oxygen; 4, admitted to hospital but requiring supplemental oxygen; 5, admitted to hospital requiring high-flow nasal cannula (HFNC), non-invasive mechanical ventilation (NIV), or both; 6, admitted to hospital requiring extracorporeal membrane oxygenation, invasive mechanical ventilation (IMV), or both; and 7, death.

In this study, patients in category 3 are referred to as “moderate patients”. Category 4 members are referred to as “severe patients”, while category 5 and 6 are referred to as “critical patients” hereafter.

Plasma and PMBC isolation

Venous blood was collected from participants and processed within 12 h to isolate plasma and peripheral blood mononuclear cell (PBMCs). Plasma was separated by centrifugation at 300 xg for 10 minutes and stored at -80°C until testing. PBMCs were isolated from blood using Ficoll-Paque PLUS (GE Healthcare, Chicago, IL) according to the manufacturer’s instructions. Isolated PBMCs were frozen in 90% heat-inactivated fetal bovine serum (FBS, Hyclone, Northbrook, IL) supplemented with 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA), and stored in liquid nitrogen before analysis.

Enzyme-linked immunosorbent assay (ELISA)

Titres of IgA, IgM, and IgG antibodies against the N, S, and RBD of SARS-CoV-2 were evaluated using the enzyme-linked immunosorbent assay (ELISA). Briefly, 20 ng of N, S, and RBD protein (Sino

Biological, Beijing, China) were used as coating protein, respectively. Plasma samples were diluted 1/400 with 0·5% bovine serum albumin (BSA) and incubated for 1 h at 37°C. After washing, horseradish peroxidaseconjugated goat anti-human Fc5μ fragment specific polyclonal IgM (Jackson ImmunoResearch, West Grove, PA, USA), rabbit anti-human α chain specific polyclonal IgA (Jackson ImmunoResearch), and goat anti-human Fc specific polyclonal IgG (Sigma Aldrich, St Louis, MO, USA) antibodies were added to the plates at a dilution of 1/60 000 with 0·5% BSA. After 1 h of incubation at 37°C, the plates were washed and developed with 100 μL substrate solutions A (3,3',5,5'-tetramethylbenzidine) and B (hydrogen peroxide) in each well (Wantai Biotech Corp, Beijing, China). The reaction was stopped by adding 50 μL of 2 M sulfuric acid. Optical density at 450 nm (OD450) was determined with a multifunctional microplate reader SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). Cut-off values were determined by calculating the mean absorbance at 450 nm of negative plasma plus 3-fold SD values, which were 0·30, 0·24, 0·20 for IgM, 0·2, 0·26, and 0·2 for IgA, 0·2, 0·2, and 0·2 for IgG against SARS-CoV-2 N, S and RBD respectively.

Microneutralization assay

NAbs were assessed on Vero cells (ATCC, Manassas, VA, CCL-81) infected with SARS-CoV-2 (IPBCAMS-WH-01/2019, no. EPI_ISL_402123, Wuhan strain hereafter), D614G, Beta, and Delta variants (all these strains were isolated from COVID-19 patient respiratory tract samples in biosafety level 3 laboratory by Institute of Pathogen Biology, Chinese Academy of Medical Sciences) using a microneutralization assay. A serial two-fold dilution of serum samples (starting at 1:10) was preincubated with SARS-CoV-2 at 100 50% tissue culture infective doses for 2 h at 37°C, and the virus-serum mixture was added to Vero cells and incubated for 1 h. The cytopathic effect was assessed 5 days after incubation. Four duplicate wells were used for each serum dilution. Neutralising antibody titres were calculated using the Reed-Muench method. Viral back-titration was done, and serum samples known to be positive for neutralising antibodies were used as a positive control in each test. The cut-off for a positive NAb titre was 1/10.

Peptide synthesis

A total of 347 15- to 18-mer peptides that overlap by 10 amino acid residues and span the S, N, membrane (M), envelope (E), ORF3a, ORF6, ORF7a, and ORF8 proteins of SARS-CoV-2,[1] and a total of 158 18-mer peptides that overlap by 10 amino acid residues targeting S protein of Beta variant were synthesized (purity >90%; Sangon Biotech, Shanghai, China). The pool contained cytomegalovirus (CMV), Epstein-Barr virus (EBV), and Influenza viruses (IFV) specific epitopes (CEF peptide pool) was used as a control.[2]

***Ex-vivo* ELISpot assay**

Ex-vivo Interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) assays were performed using cryopreserved PBMCs with a Human IFN-γ ELISpot kit (Mabtech, NS, Sweden) following the manufacturer's instructions. Briefly, cryopreserved PBMCs were thawed and washed once with the RPMI-1640 medium containing 10% FBS and rested overnight prior to assay. Human IFN-γ pre-coated plates were washed with phosphate-buffered saline (PBS), and blocked with culture media containing 10% FBS for 30 min. Total 2×10^5 PBMC per well were stimulated in duplicates with overlapping peptide pools at a final concentration of 2 μg/ml for 24 h. DMSO were used as negative controls. Phorbol myristate acetate (PMA)/ionomycin (Multi Science, Hangzhou, China) was used as positive control.

Spots were counted using an AID ELISPOT Reader System (AID GmbH, Strasberg, Germany). Mean spots of the negative control wells were subtracted from the test wells to quantify the intensity of antigen-specific T cell responses, and the results were presented as Spot Forming Unit per 10^6 PBMC (s.f.u./10^6 PBMC). T cell responses were considered positive if the mean spot count was ≥ 3-fold higher than the mean spot of the negative control and ≥20 s.f.u./10^6 PBMCs. If negative control wells had >100 s.f.u. /10^6 PBMC or positive control wells had <1000 s.f.u/10^6 PBMC, the results were excluded from further analysis.

PBMC *in vitro* expansion culture

For *in vitro* culture, 1×10^6 PBMCs were plated in 24-well plates and pulsed as a pellet for 1 h at 37 °C with 10 μM of SARS-CoV-2 peptides and cultured with fresh PRMI 1640 (Gibco, Thermo Fisher Scientific, Massachusetts, US) supplemented with 10% human AB serum and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO), under addition of 100 U/ml recombinant human interleukin (rIL-2, PeproTech, Rocky Hill, NJ). During culturing, half of the medium was changed on days 3, 5, and 7. The cells were subcultured when needed. Expanded PBMCs were analyzed by ELISpot assay and intracellular cytokine staining (ICS).

ELISpot assay using expanded PBMCs

Expanded PBMCs were washed once with the RPMI-1640 medium containing 10% FBS and rested overnight prior to assay. IFN-γ ELISpot assays were performed using a Human IFN-γ ELISpot kit (Mabtech, NS, Sweden) following the manufacturer's instructions, as ex-vivo ELISpot assay

Intracellular cytokine staining (ICS)

PBMCs were incubated with pooled peptides at a final concentration of 10 μg/mL in the presence of 1 μg/ml monoclonal antibodies CD28 and CD49d for 1 h, and then with brefeldin A (GolgiPlug, Biolegend, San Diego, CA) and monensin (GolgiStop, Biolegend) for an additional 5 h. Dead cells were labeled using BD Horizon Fixable Viability Stain 510 (BD Biosciences). Surface staining was performed using PerCP-Cy5.5-anti-CD3, BV650-anti-human CD4, PE-Cy7-anti-human CD8 (Biolegend). After incubation with fixation/ permeabilization stain buffer (Invitrogen, Carlsbad, CA), cells were immunostained using BV421-anti-IFNγ, BV711-anti-TNFα, APC-anti-IL-2 (Biolegend). Information about reagents used for intracellular cytokine staining (ICS) are listed in appendix pp 9–10. No peptide stimulation was used as a negative control for each condition. Specific cytokine responses were calculated by subtracting the background activation before further analysis. T cells exposed to PMA/ionomycin served as positive controls. All samples were acquired on a BD LSRFortessa (BD Biosciences) flow cytometer and analyzed using FCS Express 7 (De NOVO software, Pasadena, CA). Single-stained CompBeads (BD Biosciences) or single-stained PBMCs were used for compensation. Unstained PBMCs were used for assessing autofluorescence.

References

1. Peng Y, Mentzer AJ, Liu G, et al. Broad and strong memory CD4(+) and CD8(+) T cells induced by SARS-CoV-2 in UK convalescent individuals following COVID-19. *Nat Immunol* 2020; **21**(11): 1336-45.

2. Currier JR, Kuta EG, Turk E, et al. A panel of MHC class I restricted viral peptides for use as a quality control for vaccine trial ELISPOT assays. *J Immunol Methods* 2002; **260**(1-2): 157-72.

Supplementary table 1. Characteristics of enrolled patients at baseline

	Total (n=1096)	Moderate (n=289)	Severe (n=734)	Critical severe (n=73)
Age, years	58·0 (48·0-65·0)	59·0 (48·0-66·0)	58·0 (48·-65·0)	53·0 (47·0-64·0)
Sex				
Men	587 (53·6%)	146 (50·5%)	393 (53·5%)	48 (65·8%)
Women	509 (46·4%)	143 (49·5%)	341 (46·5%)	25 (34·2%)
Cigarette smoking				
Never-smoker	991/1094 (90·6%)	266 (92·0%)	664/732 (90·7%)	61 (83·6%)
Current smoker	73/1094 (6·7%)	15 (5·2%)	48/732 (6·6%)	10 (13·7%)
Former smoker	30/1094 (2·7%)	8 (2·8%)	20/732 (2·7%)	2 (2·7%)
Comorbidities				
Hypertension	321 (29·3%)	86 (29·8%)	205 (27·9%)	30 (41·1%)
Diabetes	143 (13·0%)	42 (14·5%)	91 (12·4%)	10 (13·7%)
Cardiovascular diseases	76 (6·9%)	26 (9·0%)	41 (5·6%)	9 (12·3%)
Cerebrovascular diseases	29 (2·6%)	7 (2·4%)	22 (3·0%)	0 (0.0%)
Malignant tumor	31 (2·8%)	5 (1·7%)	24 (3·3%)	2 (2·7%)
Chronic obstructive pulmonary disorder	17 (1·6%)	2 (0·7%)	15 (2·0%)	0 (0.0%)
Chronic kidney disease	14 (1·3%)	3 (1·0%)	9 (1·2%)	2 (2·7%)
Systolic blood pressure ⩾140 mm Hg	250 (22·8%)	75 (26·0%)	166 (22·6%)	9 (12·3%)
Diastolic blood pressure ⩾90 mm Hg	242 (22·1%)	70 (24·2%)	158 (21·5%)	14 (19·2%)
Length of hospital stay, days	13·0 (9·75-19·0)	11·0 (8·0-16·0)	14·0 (10·0-19·0)	42·0 (25·0-52·0)
ICU admission	45 (4·1%)	0 (0.0%)	19 (2·6%)	26 (35·6%)
Length of ICU stay, days	14·0 (6·0-28·0)	NA	7·0 (2·5-18·0)	21·5 (10·0-39·3)

Date are n (%), n/N (%), or median (IQR). ICU = intensive care unit. NA= not applicable.

Supplementary table 2. Participant demographic information for recruited recovered COVID-19 patients for antibody and T cell responses analysis

Item	Detection methods	Sample No.			
		Total	Moderate	Severe	Critical
SARS-CoV-2 N-, S-, RBD-IgM, IgA, IgG	ELISA	1096	289	734	73
Neutralising antibody	MN	141	48	57	36
IFN-γ	ELISpot	80	32	24	24
IL-2, IFN-γ, TNF-α	ICS	92	35	29	28

ELISA=Enzyme-linked immunosorbent assay; MN=Microneutralization assay; ELISpot=Enzyme-linked immunospot; ICS=Intracellular cytokine staining.

Supplementary table 3. Seropositivity of IgM, IgA, and IgG against SARS-CoV-2 N, S, and RBD in patients 12 months after initial infection of SARS-CoV-2.

	IgG	IgA	IgM
N	82·0% (899/1096)	3·5% (38/1096)	1·0% (11/1096)
S	95·2% (1043/1096)	4·5 % (49/1096)	2·6% (28/1096)
RBD	94·2% (1032/1096)	2·5% (27/1096)	2·4% (26/1096)
p value	<0·0001	0·037	0·017

N=nucleoprotein; S=Spike; RBD= receptor binding domain.

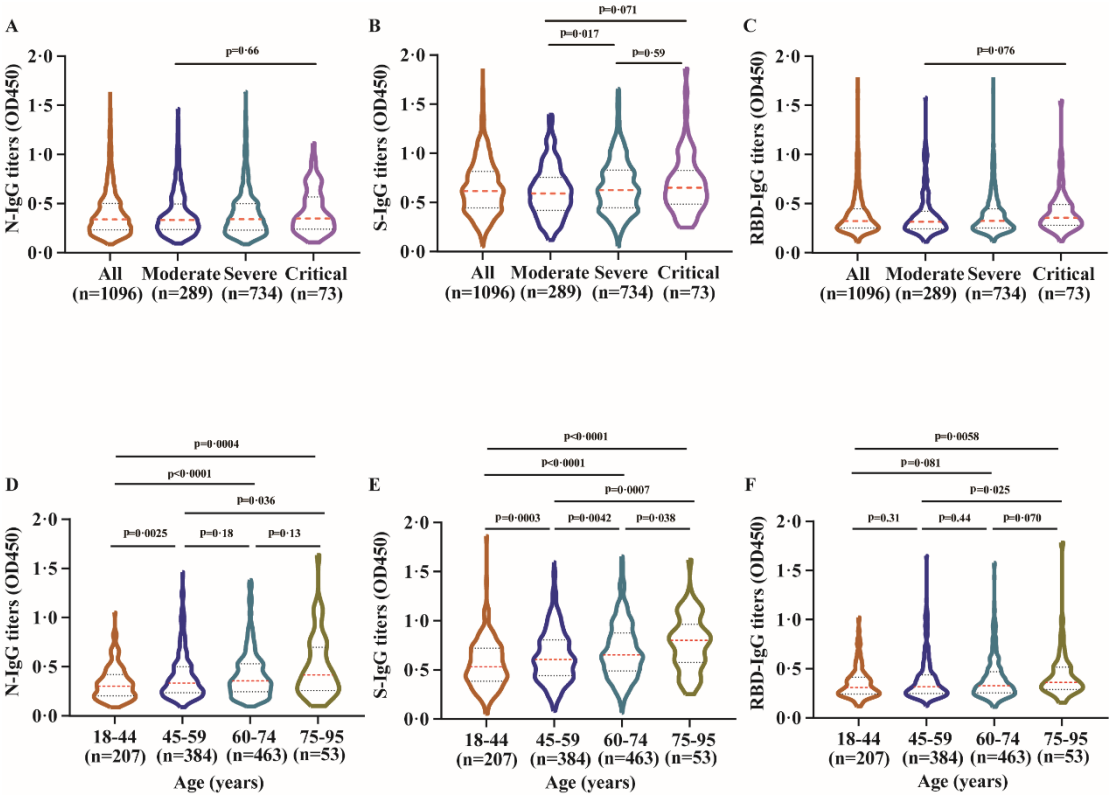
Seropositivity were compared using the χ^2 test.

Supplementary table 4. Reagents used in this study

Reagent	Information	Source	Identifier
Recombinant proteins	**Position**		
SARS-CoV-2 (2019-nCoV) Spike S1+S2 ECD-His Recombinant Protein	Val 16-Pro1213	Sino Biological	40589-V08B1
SARS-CoV-2 (2019-nCoV) Spike RBD Recombinant-His Protein	Arg319-Phe541	Sino Biological	40592-V08B
SARS-CoV-2 (2019-nCoV) nucleoprotein-His Recombinant Protein	Met1-Ala419	In house preparation	NA
Antibodies	**Clone**		
Horseradish peroxidase conjugated goat anti-human Fc5μ fragment specific polyclonal IgM	NA	Jackson ImmunoResearch	109-035-043
Horseradish peroxidase conjugated goat rabbit anti-human α chain specific polyclonal IgA	NA	Sigma Aldrich	A0295
Horseradish peroxidase conjugated goat anti-human Fc specific polyclonal IgG	NA	Sigma Aldrich	A1070
PerCP-Cy5.5- anti-CD3	SK7	Biolegend	344808
BV650-anti-human CD4	RPA-T4	Biolegend	300536
PE-Cy7-anti-human CD8	SK1	Biolegend	344712
BV421-anti-IFNγ	4S.B3	Biolegend	502532
BV711-anti-TNFα	MAb11	Biolegend	502940
APC-anti-IL-2	MQ1-17H12	Biolegend	500310
Live/Dead buffer			
BD Horizon Fixable Viability Stain 510	NA	BD Biosciences	564406
Stimulates	**Clone**		

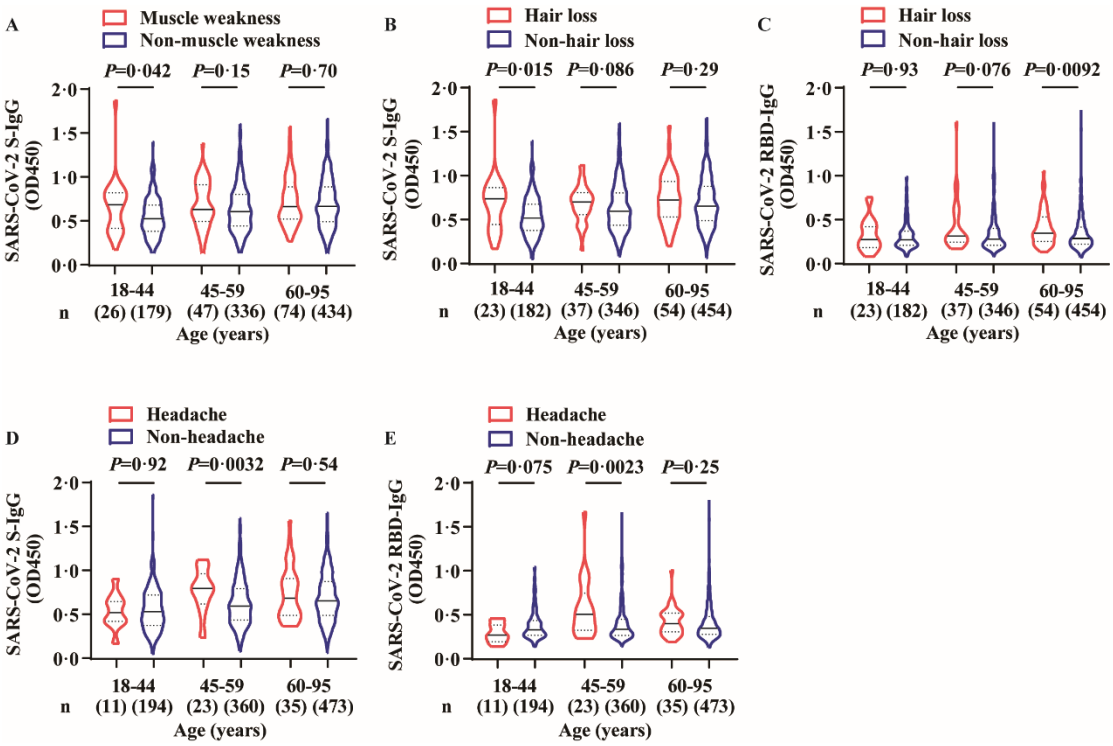
Purified NA/LE Mouse Anti-Human CD3	HIT3a	BD Biosciences	555336
Purified mouse anti-human CD28	CD28.2	Biolegend	302902
Purified anti-human CD49d	9F10	Biolegend	304302
Brefeldin A Solution (1,000X)	NA	Biolegend	420601
Monensin Solution (1,000X)	NA	Biolegend	420701
PMA/Ionomycin Mixture (250×)	NA	Multi Science	CS1001
Hunan Interleukin-2	NA	PeproTech	200-02
Foxp3 / Transcription Factor Staining Buffer Set	NA	Invitrogen	00-5523-00
Fixation/ Permeabilization concentrate (4×)	NA	Invitrogen	00-5523-00
Fixation/ Permeabilization Diluent	NA	Invitrogen	00-5523-00
Permeabilization buffer (10×)	NA	Invitrogen	00-5523-00

Supplementary figure 1. Plasma IgG antibodies against SARS-CoV-2 in individuals recovered from COVID-19



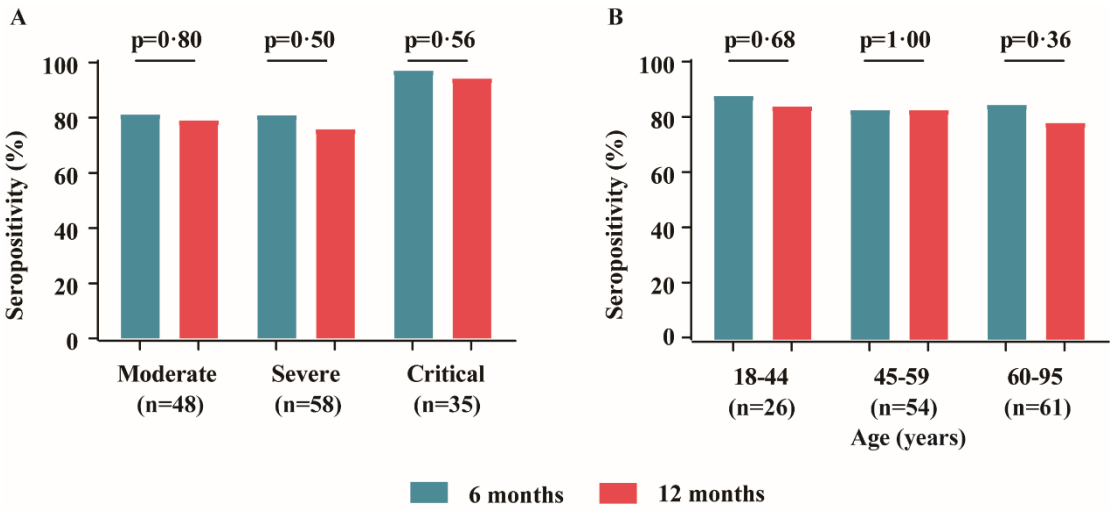
(A–C) IgG titres against SARS-CoV-2 N (A), S (B), and RBD (C) proteins in moderate, severe, and critical COVID-19 recovered patients 12 months after infection. (D–F) The IgG titres against SARS-CoV-2 N (D), S (E), and RBD (F) proteins in recovered patients aged 18–44, 45–59, 60–74, and 75–95 years old 12 months after infection. The sample size of different disease severity and age groups was included in the figures. Red and black dotted lines in violin denote the median and interquartile range of antibody titres, respectively. The comparisons of IgG titers were performed using Kruskal-Wallis test for multiple testing. OD450=optical density at 450 nm.

Supplementary figure 2. SARS-CoV-2 specific IgG titer in participants with sequelae symptoms



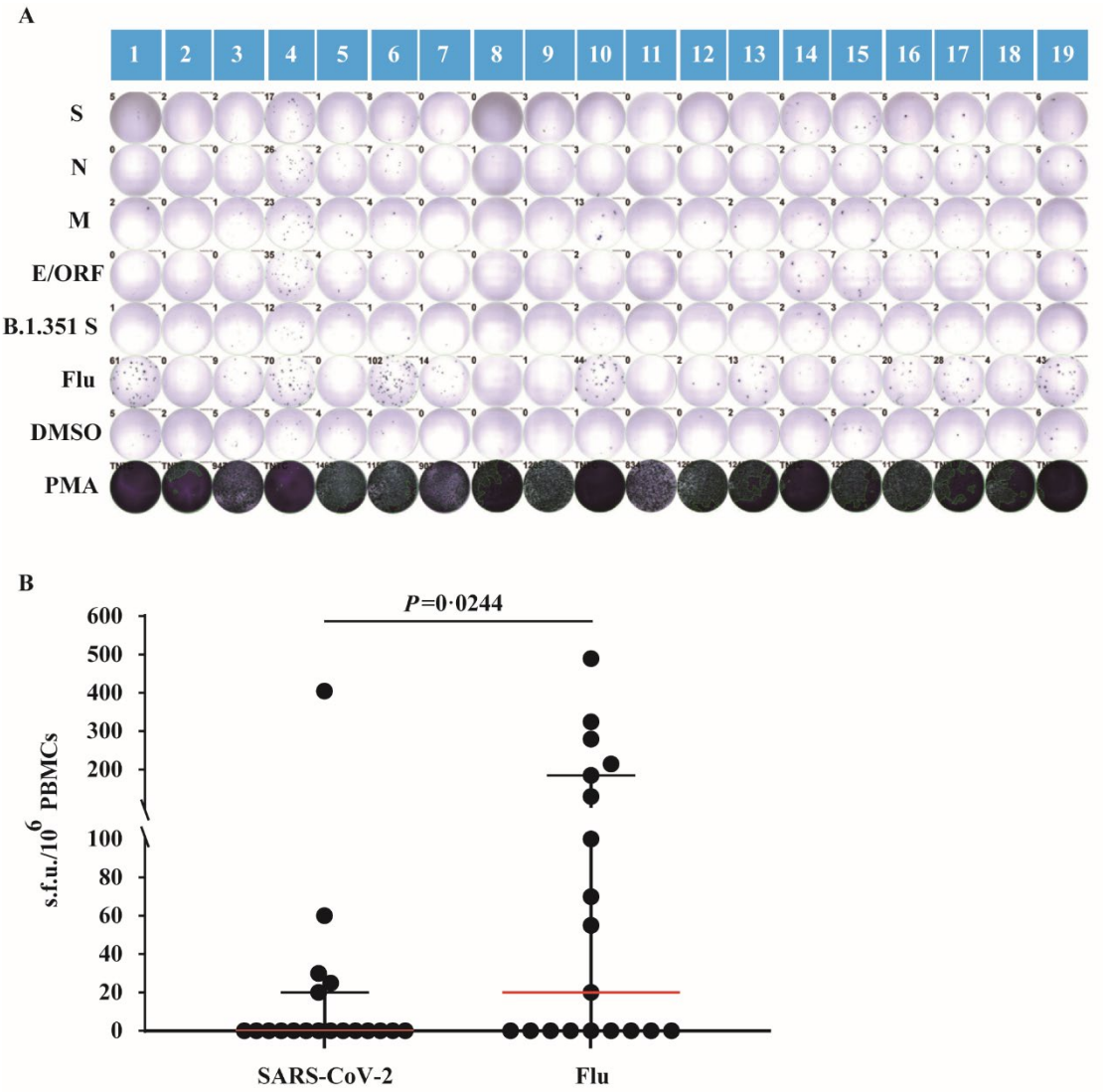
(A) SARS-CoV-2 S-IgG titres in different age groups comparing patients with or without muscle weakness. (B–C) Comparison of SARS-CoV-2 S-IgG (B) and RBD-IgG (C) titres in different age groups in patients with or without hair loss. (D–E) Comparison of SARS-CoV-2 S-IgG (D), and RBD-IgG (E) titres in different age groups with or without headache. The sample size was included in the figures. Data are shown as medians with interquartile ranges. Non-parametric Mann–Whitney test was used for comparison of antibody titres.

Supplementary figure 3. Seropositivity of neutralising antibodies against SARS-CoV-2 Wuhan strain 6- and 12- months after SARS-CoV-2 infection



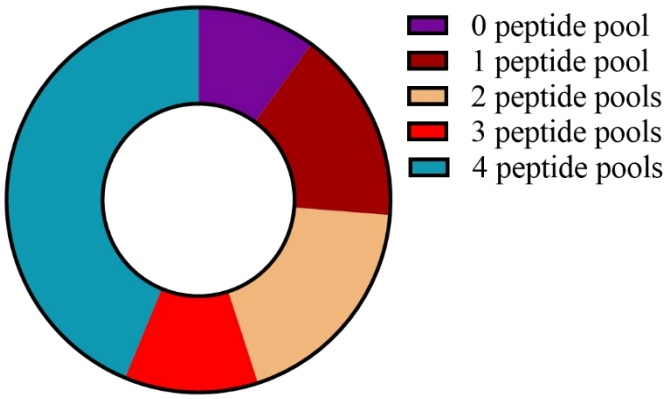
Seropositivity of neutralising antibodies against SARS-CoV-2 Wuhan strain in moderate, severe, and critical COVID-19 patients (A) and across different age groups (B) at 6- and 12-months after infection.

Supplementary figure 4. Magnitude of T cell responses against SARS-CoV-2 antigens in unexposed healthy controls



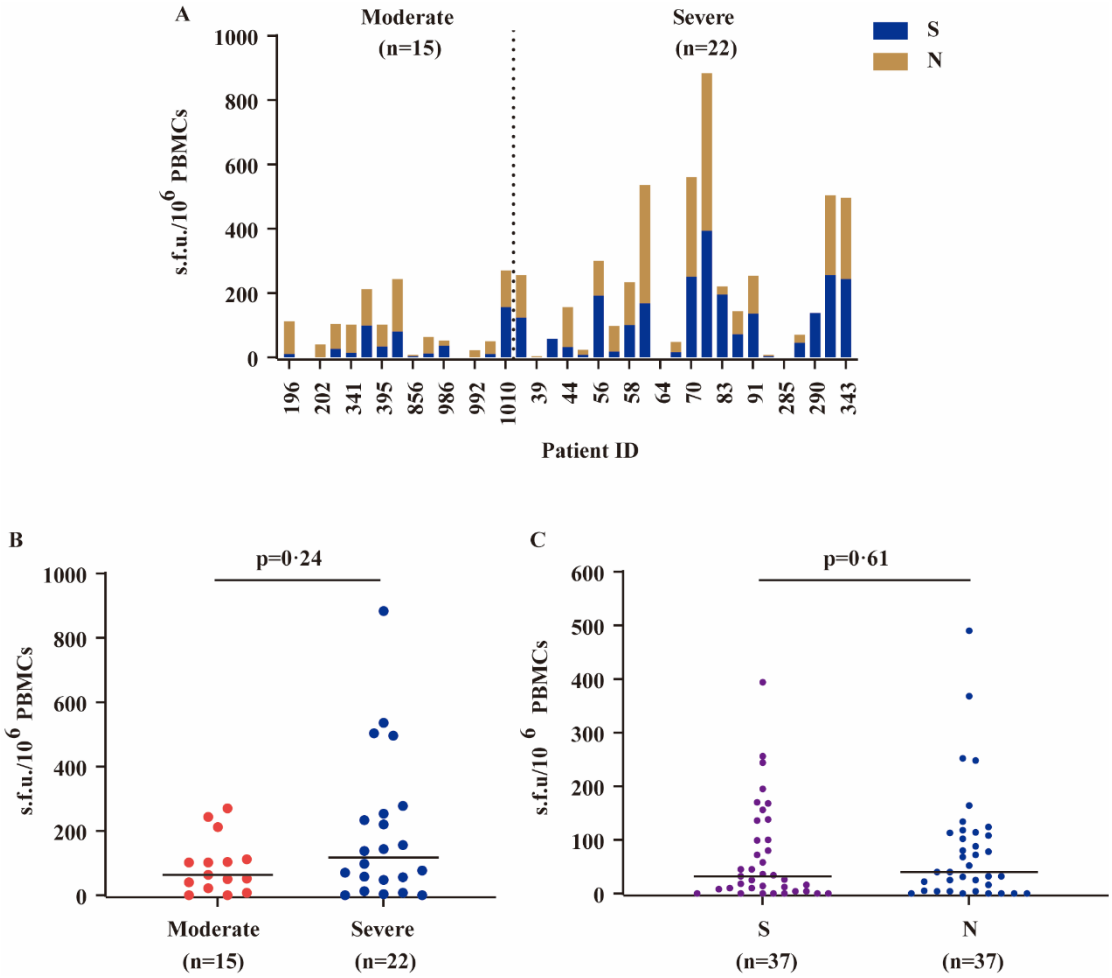
(A) IFN-r ELISpot plate from nineteen healthy control participants without prior SARS-CoV-2 infection. Each individual has been tested with S pools, N pools, M pools, E/ORF pools, Beta variant S pools, known CD8+ T cell epitope pools of human influenza (Flu). DMSO with no peptide was used as a negative control; PMA/ionomycin was used as a positive control. (B) Magnitude of T cell responses against SARS-CoV-2 peptide pools and Flu peptide pool in healthy controls without a COVID-19 history (n=19). Data are presented as median with interquartile range. Non-parametric Mann–Whitney test was used for comparison of magnitude of T cell responses.

Supplementary figure 5. Memory T cell responses specific to SARS-CoV-2 peptides measured by IFN-γ ELISpot



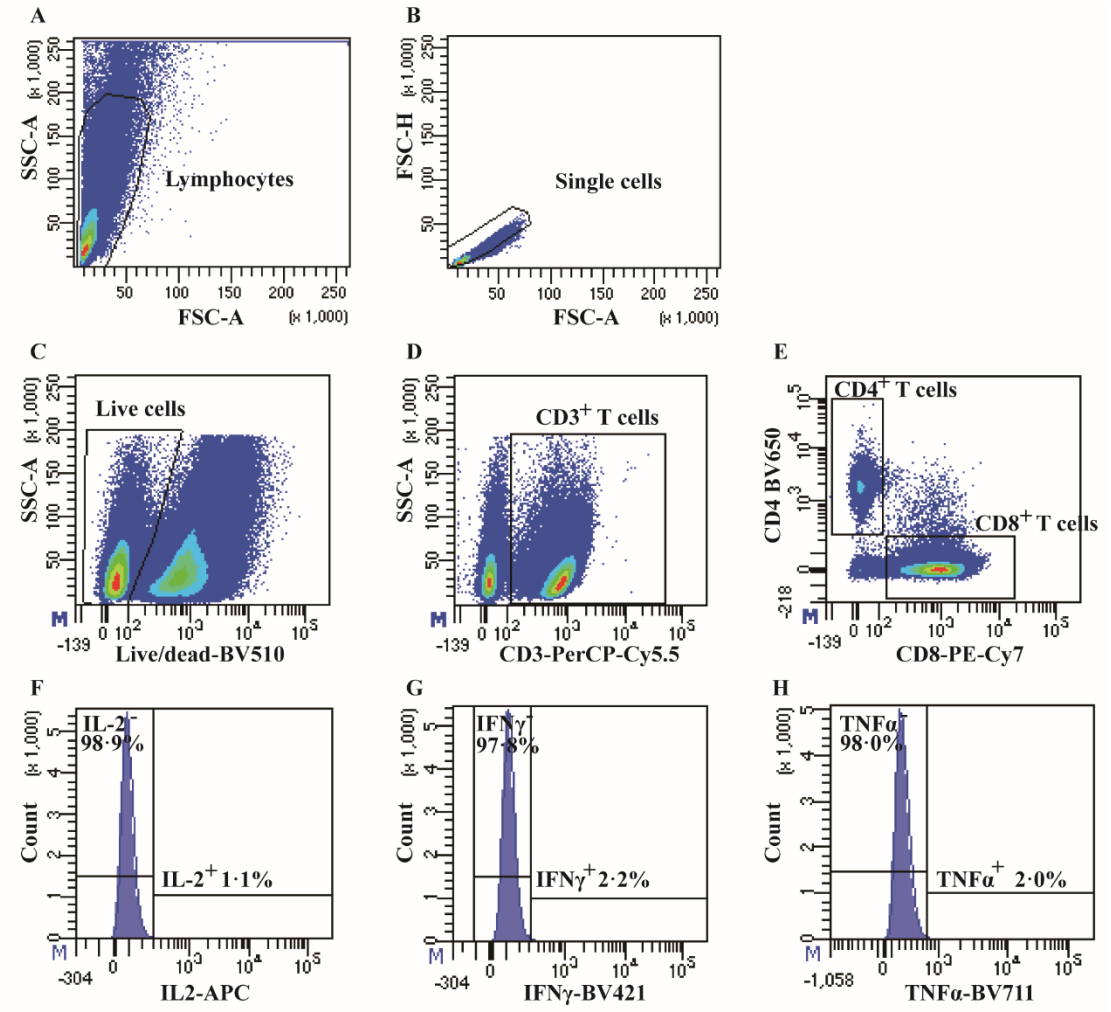
Pie charts representing the relative proportions of T cell responses producing IFN-γ against zero, one, two, three, or four peptide pools (S-, N- M-, and E/ORF) (n=80).

Supplementary figure 6. Memory T cell responses specific to SARS-CoV-2 peptides measured by *ex-vivo* IFN-γ ELISpot



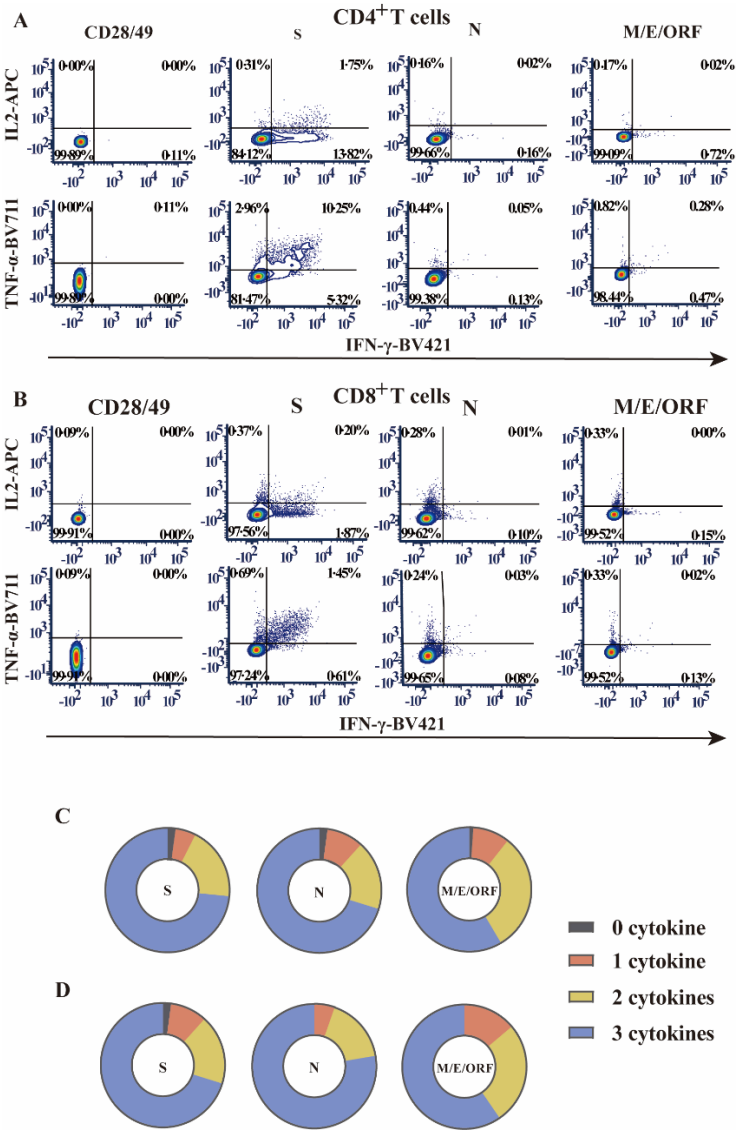
(A) Magnitude of IFN-γ T cell responses for each individual. Each bar shows the total T cell responses of each individual specific to the SARS-CoV-2 spike and N peptide pools tested. Each colored segment represents the source protein corresponding to peptide pools eliciting IFN-γ T cell responses. (B) Magnitude of IFN-γ responses in individuals with different disease severity. (C) Magnitude of IFN-γ against S and N peptide pools. The sample size was included in the figures. The solid lines in (B) and (C) denote the median of magnitude of IFN-γ responses. Statistical differences were performed with the Mann-Whitney test. The sample size was included in the figures.

Supplementary figure 7. Gating strategy for flow cytometry analysis



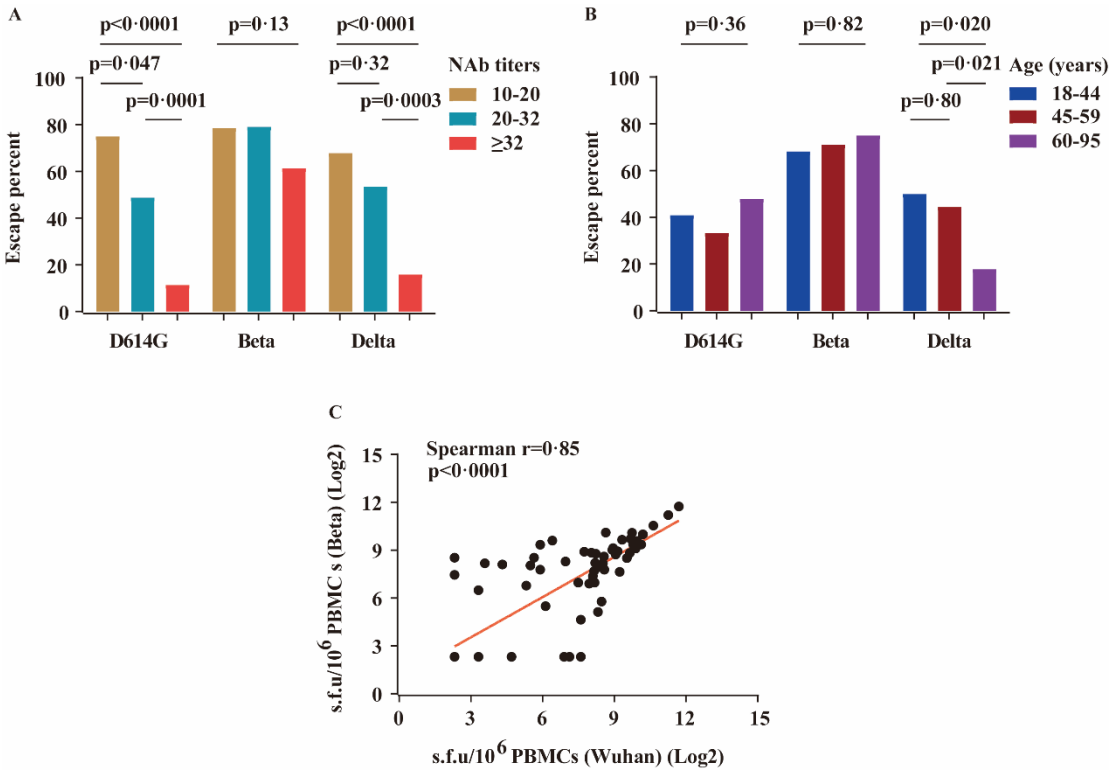
(A–E) Gating for $CD4^+$/$CD8^+$ T cells. Cells were gated on single cells by a forward side scatter gate, followed by CD3/ CD4/CD8 gating excluding dead cells, $CD14^+$, $CD19^+$, and $CD16^+$ cells. (F–H) Gating for IL-$2^{+/-}$, IFN-$\gamma^{+/-}$, and TNF-$\alpha^{+/-}$. Population was based on corresponding negative controls.

Supplementary figure 8. Functional characteristics of SARS-CoV-2-specific T cells in recovered COVID-19 patients



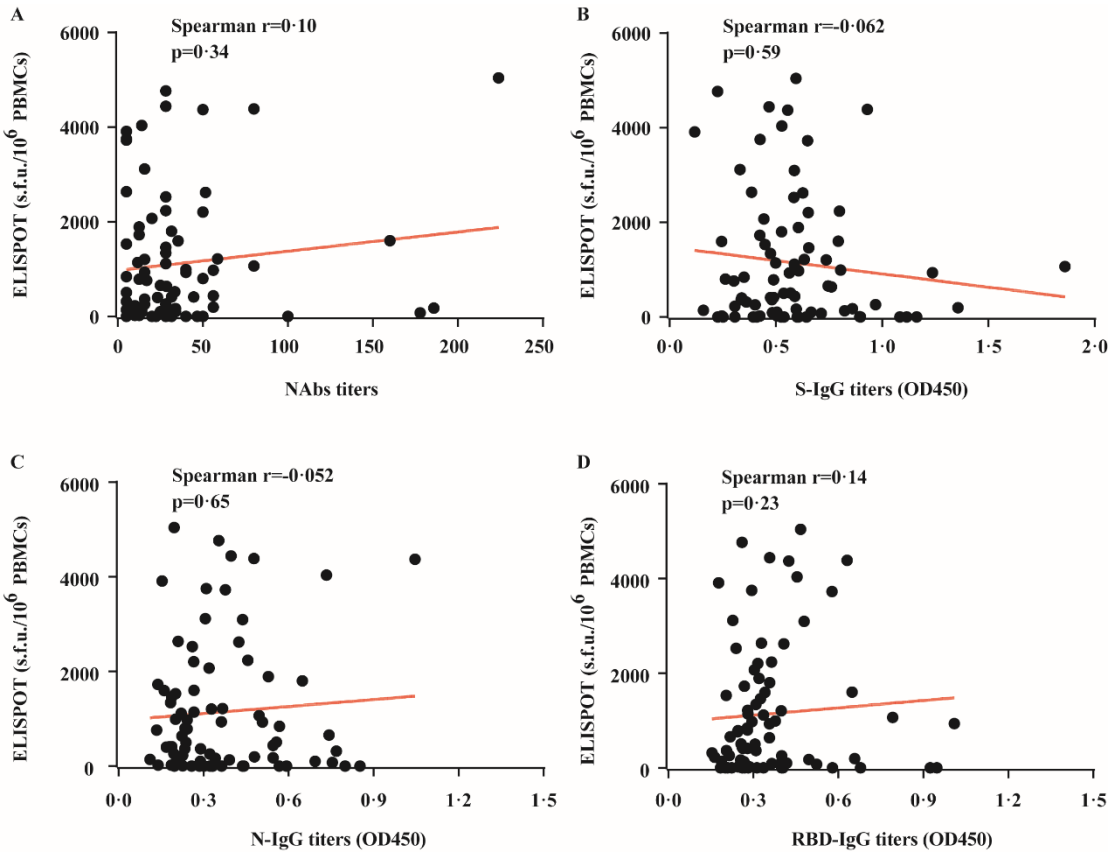
(**A, B**) Flow cytometric plots representing CD4$^+$T cells (A) and CD8$^+$T cells (B) expressing IFN-γ (x axis), IL-2 (y axis) and TNF (y axis) upon stimulation with the respective SARS-CoV-2 peptide pools. Samples from 35 moderate, 29 severe, and 28 critical recovered COVID-19 patients were assayed. Numbers indicate percentages in the drawn gates. (**C, D**) Pie charts representing the relative proportions of S-, N- and M/E/ORF-specific CD4$^+$ (C) and CD8$^+$ (D) T cells producing zero （black), one (red), two (yellow) or three cytokines (blue) (out of IFN-γ, TNF and IL-2) in 92 recovered patients.

Supplementary figure 9. Humoral and cellular immune responses to the SARS-CoV-2 Wuhan strain, D614G variant, Beta variant, and Delta variant



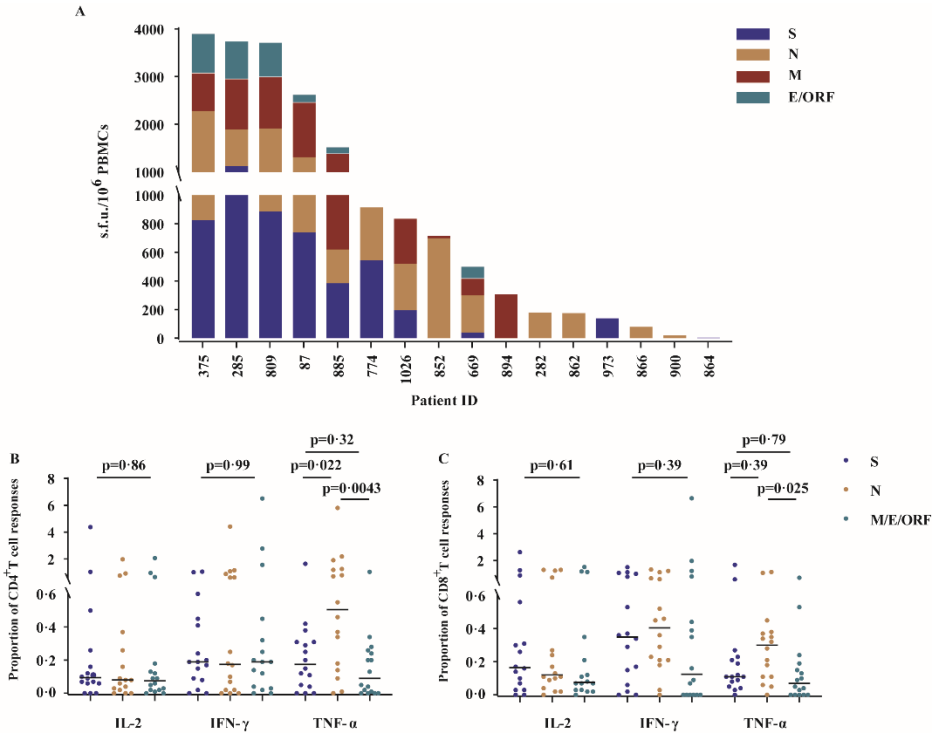
The escape percentage of D614G, Beta, and Delta SARS-CoV-2 variants from neutralising antibodies against SARS-CoV-2 Wuhan strain in patients with different antibody titres (A) and different age groups (B). (C) The correlation of magnitude of IFN-γ T cell responses between SARS-CoV-2 Wuhan strain and Beta SARS-CoV-2 variant S peptide pools in 80 recovered patients. The escape percent of D614G, Beta and Delta from neutralising antibodies was compared using the χ^2 test. Spearman correlation analysis was performed for the correlation analyses.

Supplementary figure 10. Correlation of T cell responses with SARS-CoV-2 antibodies



The correlation between IFN-γ T cell responses measured by ELISpot and neutralising antibodies (A), S-IgG (B), N-IgG (C), and RBD-IgG (D) titres (n=80). Spearman correlation analysis was performed for the correlation analyses.

Supplementary figure 11. SARS-CoV-2 memory T cell responses in neutralising antibody negative individuals



(A) Magnitude of IFN-γ T cell responses in ELISpot for each individual who lost neutralising antibody responses (n=16). Each bar shows the total T cell responses in each individual specific to all the SARS-CoV-2 protein peptide pools tested. Each colored segment represents the source protein corresponding to peptide pools eliciting IFN-γ T cell responses. (B–C) The distribution of multi-functional cytokines against different peptide pools in SARS-CoV-2-specific CD4+ (B) and CD8+ T cells (C) for each individual who lost neutralising antibody responses (n=16). The lines denote the median of proportion of T cell responses. Multiple comparisons were performed using the Kruskal-Wallis test.